

Glutamatergic regulation prevents hippocampal-dependent age-related cognitive decline through dendritic spine clustering

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The dementia of Alzheimer's disease (AD) results primarily from degeneration of neurons that furnish glutamatergic corticocortical connections that subserve cognition. Although neuron death is minimal in the absence of AD, age-related cognitive decline does occur in animals as well as humans, and it decreases quality of life for elderly people. Age-related cognitive decline has been linked to synapse loss and/or alterations of synaptic proteins that impair function in regions such as the hippocampus and prefrontal cortex. These synaptic alterations are likely reversible, such that maintenance of synaptic health in the face of aging is a critically important therapeutic goal. Here, we show that riluzole can protect against some of the synaptic alterations in hippocampus that are linked to age-related memory loss in rats. Riluzole increases glutamate uptake through glial transporters and is thought to decrease glutamate spillover to extrasynaptic NMDA receptors while increasing synaptic glutamatergic activity. Treated aged rats were protected against age-related cognitive decline displayed in nontreated aged animals. Memory performance correlated with density of thin spines on apical dendrites in CA1, although not with mushroom spines. Furthermore, riluzole-treated rats had an increase in clustering of thin spines that correlated with memory performance and was specific to the apical, but not the basilar, dendrites of CA1. Clustering of synaptic inputs is thought to allow nonlinear summation of synaptic strength. These findings further elucidate neuroplastic changes in glutamatergic circuits with aging and advance therapeutic development to prevent and treat age-related cognitive decline.

cognitive aging | glutamate | riluzole | neuroplasticity | dendritic spine clustering

Cognitive decline often occurs with aging in rodents (1), nonhuman primates (2), and humans (3). Memory loss (4) and executive impairment (5) are of the most functional importance, mediated primarily by the hippocampus and related areas of the medial temporal lobe and the prefrontal cortex (PFC), respectively. The neural circuits vulnerable to aging are composed of glutamatergic pyramidal neurons that furnish corticocortical connections between the association cortices as well as the excitatory hippocampal connections (2, 6). Dendritic spine changes, which appear to be the primary site of structural plasticity in the adult brain (7), occur in the pyramidal neurons of the PFC (5) and in the hippocampus (8, 9) with aging and correlate with behavioral decline. Spines form the postsynaptic component of most excitatory synapses in the cerebral cortex and are capable of rapid formation, expansion, contraction, and elimination (10, 11).

Synaptic glutamatergic activity is neuroprotective and critical for long-term potentiation (LTP) and memory formation, whereas extrasynaptic NMDA receptor activity promotes long-term depression and excitotoxicity (12, 13). There is some evidence that astrocytic glutamate transporters decrease with aging

(14, 15), and consequently reduce glutamate uptake (14, 16, 17). Reduced glutamate uptake can lead to glutamate spillover to the extrasynaptic space with electrophysiological repercussions (14). The potential use of glutamate modulators as a therapeutic target to regulate the synaptic age-related glutamatergic dysregulation in those vulnerable neural circuits remains to be further investigated.

Riluzole is a glutamate modulator that decreases glutamate release (18) and facilitates astrocytic glutamate uptake (19–21). These actions have been suggested to increase glutamate-glutamine cycling, enhancing synaptic glutamatergic activity while preventing excessive glutamate overflow to the extrasynaptic space in rodents (21, 22) and humans (23). Riluzole has also been shown to increase oxidative metabolism with mitochondrial enhancing properties (24) and to increase BDNF expression (25). We hypothesized that improved regulation of the glutamatergic synapse with the glutamate modulator riluzole would promote synaptic NMDA receptor activation while preventing extrasynaptic NMDA activity, thereby protecting against age-related cognitive decline, through induction of neuroplastic changes in the hippocampus and PFC. An important neuroplastic mechanism is clustering of dendritic spines because it significantly empowers neural circuits with nonlinear summation of synaptic inputs (26, 27) and is dependent on neuronal activity (28, 29). For this study, we focused on pyramidal neurons within CA1 and pyramidal neurons in layer 3 of the prelimbic region of medial PFC, an area where we have demonstrated age-related spine loss in middle-aged animals previously (30).

Significance

Aging is often accompanied by cognitive decline. It is of critical importance to understand the synaptic susceptibilities of the glutamatergic neural circuits to age-related cognitive decline and to intervene in this process. Maintenance of synaptic health in the face of aging is a crucially important therapeutic goal. We show that the glutamate modulator, riluzole, prevents age-related memory loss and induces clustering of dendritic spines. Clustering is a critical element of synaptic plasticity that has been previously demonstrated to increase synaptic strength. This study further elucidates neuroplastic changes in the neurocircuits vulnerable to aging and advances therapeutic development to prevent and treat age-related cognitive decline.

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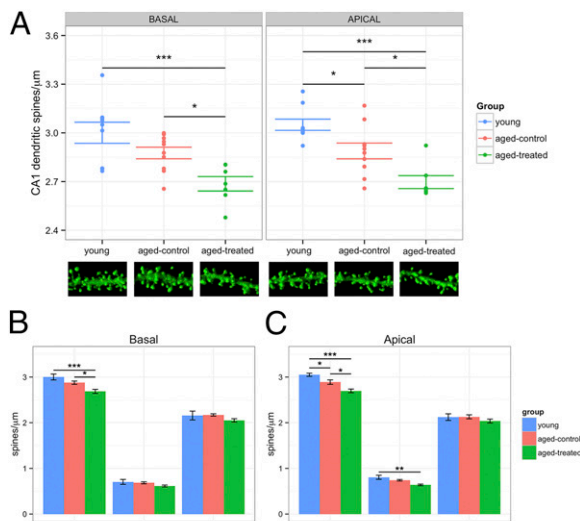


Fig. 2. Morphometric analysis of LY-filled neurons. (A) Total spine density was significantly different among groups at basal [$F(2,23) = 8.95$, $P = 0.001^{**}$] and apical [$F(2,23) = 7.22$, $P < 0.001^{***}$] dendrites. (B) In basal dendrites, a difference occurred between young and aged-treated animals ($P < 0.001^{***}$) and between aged-control and aged-treated animals ($P < 0.05^{*}$). Mushroom and thin spine subgroups in basal dendrites did not show a significant difference among groups ($P = 0.24$ and $P = 0.39$, respectively). (C) In apical dendrites, there was a significant difference between young and aged-control animals ($P < 0.05^{*}$), young and aged-treated animals ($P < 0.001^{***}$), and aged-control and aged-treated animals ($P < 0.05^{*}$). Mushroom spines were different among groups [$F(2,23) = 7.22$, $P = 0.0037^{**}$]. There was a difference between young and aged-treated animals ($P < 0.01^{**}$) but not between young and aged-control animals or aged-control and aged-treated animals. There was no significant difference among groups for thin spine subtype in apical dendrites [$F(2,23) = 0.7$, $P = 0.48$].

subgroups in basal dendrites did not show a significant difference among groups ($P = 0.24$ and $P = 0.39$, respectively) (Fig. 2B).

A one-way ANOVA comparing the three groups for total spine density in apical dendrites showed a significant difference [$F(2,23) = 7.22$, $P < 0.001^{***}$] (Fig. 2A). Tukey post hoc comparison showed a significant difference between young and aged-control ($P < 0.05^{*}$), young and aged-treated ($P < 0.001^{***}$), and aged-control and aged-treated ($P < 0.05^{*}$) animals. Mushroom spines were different among groups [$F(2,23) = 7.22$, $P = 0.0037^{**}$]. Post hoc Tukey comparison showed a difference between young and aged-treated animals ($P < 0.01^{**}$) but not between young and aged-control animals or aged-control and aged-treated animals (Fig. 2C). There was no significant difference among groups for thin spine subtype in apical dendrites [$F(2,23) = 0.7$, $P = 0.48$].

In PFC, there was not a significant change in total spine density between aged riluzole-treated and aged-control animals ($P = 0.52$, $t = 0.65$). Even when the analysis separated the different spine subtypes (thin and mushroom) and dendritic domains (apical and basal), no difference in density was found.

Thin spine density in CA1 in riluzole-treated animals showed a significant positive Pearson correlation with Y-maze performance essentially throughout the trial, including the middle at 5 min ($r = 0.89$, $P = 0.0065^{**}$) and the end at 10 min ($r = 0.81$, $P = 0.026^{*}$). In contrast, density of mushroom spines was unrelated to cognitive performance ($r = -0.23$, $P = 0.60$). Further analysis of thin spine location demonstrated that this correlation with the hippocampal-dependent Y-maze task was driven by apical thin spine density ($r = 0.81$, $P = 0.026^{*}$) (Fig. 3A) and not by basal thin spines ($r = -0.26$, $P = 0.57$) (Fig. 3E). Apical thin dendritic spine density did not correlate with Y-maze performance in aged-control animals ($r = 0.43$, $P = 0.20$) (Fig. 3B). Apical and basal mushroom spine densities were unrelated to behavioral

performance in treated animals [$r = -0.62$, $P = 0.13$ (Fig. 3D) and $r = 0.28$, $P = 0.5$ (Fig. 3F), respectively]. Apical thin spine density also correlated with Y-maze performance in young animals ($r = 0.80$, $P = 0.009^{**}$) (Fig. 3C). Errors bars are shown across dendrites and plotted for each animal. In PFC, no correlation was found between behavioral performance and spine density. These results suggest that the riluzole prevention of hippocampal-related cognitive decline during aging is due to neuroplastic changes that occur specifically for thin spines on apical dendrites.

Riluzole-Treated Rats Had an Increase in Clustering of Thin Spines That Correlated with Memory Performance and Was Specific to the Apical Dendrites of CA1.

One way by which a small decrease in synaptic density might be related to increased synaptic strength would be if it reflects increased spine clustering. Potentiation at one spine can stabilize and cause retention of that spine, along with its neighbors (33–35). Here, we measured clustering using a Euclidean distance algorithm to calculate the distance of every spine head to its nearest neighboring spine head in 3D space. Because we had observed a small change in spine density between the aged riluzole-treated and untreated animals, we normalized by density to minimize the effect of density on the distances between spine heads. We then used a cumulative distribution plot to observe the distances where there was greater variation between the aged riluzole-treated and the untreated aged animals and tested for significance using a Kolmogorov–Smirnov test with an alpha of 0.05.

To test whether the effect of riluzole elicits a spine distribution similar to what would be observed in young animals, we compared the clustering data from young, aged-control, and aged riluzole-treated animals. We found no significant difference in minimum distances between spine heads between aged-control animals and young-control animals in either the basal or apical

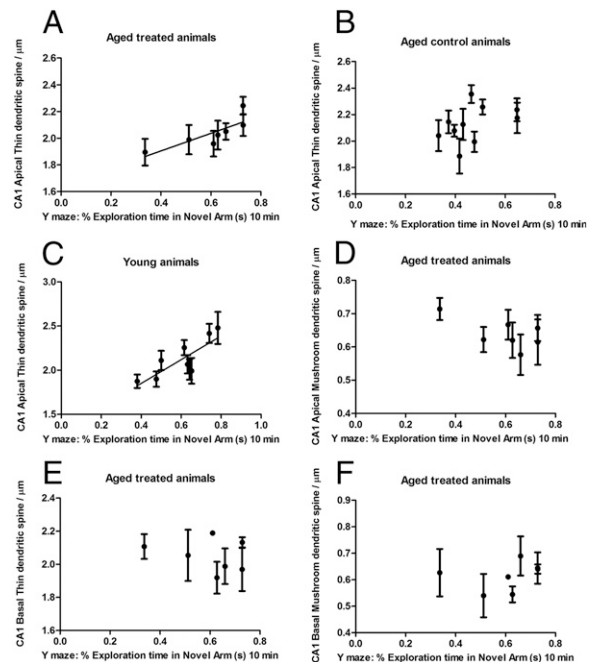


Fig. 3. Memory performance correlated with the density of thin spines on apical dendrites in CA1. Apical thin spine density in CA1 showed a significant positive Pearson correlation with Y-maze performance in aged-treated animals (A; $r = 0.81$, $P = 0.026^{*}$) and in young animals (C; $r = 0.80$, $P = 0.009^{**}$). (B) Apical thin dendritic spine density did not correlate with Y-maze performance in aged-control animals ($r = 0.43$, $P = 0.20$). (D) Apical mushroom spines were unrelated to Y-maze performance in treated animals ($r = -0.62$, $P = 0.13$). Basal thin and mushroom densities did not correlate with behavioral performance [$r = -0.26$, $P = 0.57$ (E) and $r = 0.28$, $P = 0.5$ (F), respectively].

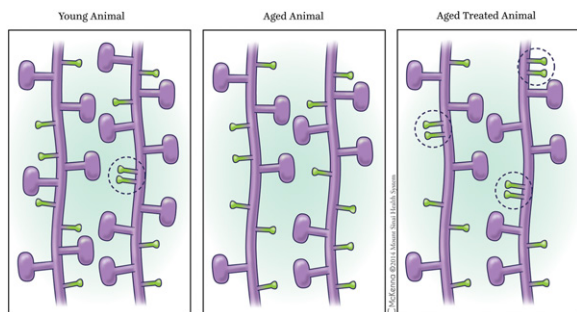


Fig. 5. Schematic representation of apical dendrites with spine clustering in CA1. Aged-treated animals presented with increased thin-thin interaction dendritic spine clustering (*Right*) in comparison to aged-control animals (*Center*) and young animals (*Left*), as shown in the encircled areas. Dendritic spine clustering has been shown to empower neural circuits with nonlinear summation of synaptic inputs. In relation to spine density, there is slightly diminished mushroom density in aged-treated animals in comparison to young animals (represented in the figure) but there is no significant thin or mushroom spine density difference between aged-control and aged-treated animals (main text). Although connectivity is altered and thin spine clustering is increased in treated animals, total thin spine density in apical dendrites is not significantly changed when comparing the three groups, as represented in the schematic figure.

(24). Future experiments could individually block each of these pathways (i.e., hippocampal infusion of glutamate transport inhibitors, use of BDNF antibody) to investigate which one(s) block the effect on behavior, further pinpointing the critical pathways.

Synaptic networks adapt to the environment through a continuous turnover process (7). Neuroplastic changes associated with learning and memory have been shown in association with formation of new spines, and pruning with elimination of spines also appears to be critical (35, 40–44). Notably, LTP on mature hippocampal neurons shows that synapse loss is perfectly counterbalanced by enlargement of the remaining excitatory synapses (45). In some cases, the increase in spine formation and elimination is similar, resulting in no marked change in spine density (46–48). As in this study, apical thin spines did not show a significant change in dendritic spine density, although there is a positive correlation between behavior and spine density and clustering. The results presented here not only mechanistically demonstrate the structural modifications associated with the behavioral enhancement produced by riluzole but also expand on the diversity and complexity of plasticity mechanisms with therapeutic relevance. It will also be interesting to investigate, when new agents are discovered to prevent cognitive decline, if clustering is a common neuroplastic compensatory mechanism in the aging brain.

In conclusion, our data suggest that riluzole may have the potential to treat or even prevent age-associated memory decline and that these findings in aged rodents can be translated to study the impact of riluzole on cognitive function in the aging human brain. This study also suggests that further exploration of novel glutamate modulators that target circuits vulnerable to aging represents a promising therapeutic strategy.

Methods

Animals. Aged, male Sprague–Dawley rats ($n = 20$, retired breeders; Harlan Laboratories) were housed at The Rockefeller University from 9 until 14–15 mo of age. Young, male Sprague–Dawley rats ($n = 10$) ordered from the same source were housed in the same facility from 3 until 4–5 mo of age. All rats were pair-housed in controlled conditions (30–50% humidity, 21 ± 2 °C, 12-h light/dark cycle). All procedures were done in agreement with the National Institutes of Health and The Rockefeller University Institutional Animal Care and Use Committee guidelines.

Treatment. Aged-treated rats ($n = 10$) had ad libitum access to riluzole solution, and aged-control ($n = 10$) and young-control ($n = 10$) rats had ad libitum access to tap water. Aged animals received riluzole or water for 17 wk. All rats had ad libitum access to food. The riluzole compound (Sigma–Aldrich, Inc.) was dissolved in tap water at a concentration of $110 \mu\text{g}/\text{mL}$, translating to $\sim 4.0 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ p.o., a dose previously tested in rats (21). To make the solution, riluzole was stirred in room temperature tap water overnight. All containers with riluzole were covered in tin foil to prevent light exposure. Fresh solutions were made every 2–3 d for the duration of treatment.

Y-Maze. The Y-maze task (31, 32) was used to measure spatial recognition memory at baseline and end point (17 wk). The task consisted of a 15-min acquisition trial, a 4-h intertrial delay, and a 10-min retention trial. During the acquisition trial, rats were placed facing away from the center of the maze in one arm (start arm) and were allowed to explore two arms (start arm and familiar arm). The third arm (novel arm) was blocked off with a Plexiglas panel identical to the other walls of the Y-maze. During the retention trial, rats were placed in the same orientation in the start arm and allowed to explore all three arms. Start, familiar, and novel arms were rotated between rats. Three different spatial cues were placed 1 foot above each arm of the maze on the curtain surrounding the apparatus (Fig. S4A). Used corncob bedding from each rat being tested was mixed and spread over the floor of the maze to reduce anxiety. Each arm of the maze was 14.5×48.26 cm. To ensure commitment to entering an arm, rat movement was recorded in a more conservative zone of 14.5×45 cm in each arm. If spatial recognition memory is intact, rats should explore the novel arm more than the familiar arm during the retention trial, given their natural tendency to explore novel environments. Extent of exploration was assessed in terms of time(s) spent in arms, number of entries into arms, latency(s) to enter arms for the first time, and arm entered first. See Table S1 and SI Methods for further methods on behavior.

Confocal Laser Scanning Microscopy and NeuronStudio Spine Morphological Analysis.

Spine imaging and analysis were conducted according to Bloss et al. (30). Concentric circles in increments of $50 \mu\text{m}$ were drawn over the 25-fold trace of the neuron from the center of the soma to provide a systematic sampling of proximal ($100 \mu\text{m}$), intermediate ($150 \mu\text{m}$), and distal ($200 \mu\text{m}$) dendritic segments. A random subset of six to eight neurons was imaged for each animal. When possible, two segments at each distance from the soma in the apical tree per neuron and two segments at each distance from the soma in the basal tree per neuron (~ 8 – 12 segments) were imaged using a Zeiss 510 confocal microscope equipped with an argon laser, Zeiss oil-immersion objective (magnification of $40\times$ and N.A. of 1.4), and ZEN software for laser scanning microscopy (49). Segments from dendritic branches had to satisfy the following criteria: (i) located within a depth of $100 \mu\text{m}$ from the surface of the section because of the limited working distance of the objective, (ii) parallel to or at acute angles relative to the coronal surface of the section to allow for unambiguous identification of spines, (iii) segments had no overlap with other branches that would obscure visualization of spines, (iv) dendrites had to be terminating rather than bifurcating, and (v) dendrite image had to be captured at least $10 \mu\text{m}$ from both the origin and the tip.

Conditions such as laser excitation (458 nm), fluorescence emission capture (505 – 530 nm), pinhole size (1 Airy unit), speed ($3.2 \mu\text{s}$ per pixel), and frame average (two frames per z-step) were held constant throughout. Confocal z-stacks were taken using a Zeiss objective (magnification of $40\times$ and N.A. of 1.4) with a digital zoom of 4.0, a z-step size of $0.1 \mu\text{m}$, and a pixel resolution of 512×512 , yielding an image with pixel dimensions of $0.05 \times 0.05 \times 0.1 \mu\text{m}$. Z-stacks were deconvoluted using AutoDeblur software to improve voxel resolution and reduce optical aberration along the z axis. Images were then imported and analyzed using custom-built NeuronStudio software (50). Analysis on Neuronstudio was performed in a semiautomated manner. In short, the settings for the automated analysis were 10% threshold correction, 350 voxels for minimum stubby size, five voxels for minimum non-stubby size, 0.8 neck ratio, 2:1 thin ratio, and 0.35 for mushroom size, with review and correction by the user in regard to spine location, subtype, and existence.

Dendritic Spine Clustering Analysis. Spine clustering was calculated utilizing a Euclidean distance algorithm in three dimensions using MATLAB 2010a (MathWorks). In brief, the distance between each spine head and its nearest neighboring spine head was collected and then normalized by the density of spines per dendrite. The distances were plotted as a cumulative distribution, and significance was tested using the Kolmogorov–Smirnov test, a

nonparametric test for similarity between continuous, 1D probability distributions. These tests were performed on all the dendrites of the PFC and CA1, as well as on divisions within each region of spine subtype (apical and basal). The Euclidean distance was defined as follows:

$$d(p, q) = d(q, p) = \sqrt{(q_1 - p_1)^2 + (q_2 - p_2)^2 + \dots + (q_n - p_n)^2} = \sqrt{\sum_{i=1}^n (q_i - p_i)^2}.$$

To test correlation of behavior with spine clustering individually, each set of spine head distances was fit to a normal distribution by animal. A cutoff was chosen between 0.6 and 0.9 based on the distances that had been found to be significantly different between the drug-treated and control animals. The area within this range was calculated by standardizing the normal distribution

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and converting the z-score obtained from the standardization to a cumulative probability score. The cumulative probability score allows for analysis of the likelihood of randomly choosing a spine with a normalized distance between 0.6 and 0.9. This value was then correlated to the animals' performance in the Y-maze.

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